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14. ABSTRACT

The major goal of this project is to optimize the strategies for using radioactive strontium for palliation of bone pain due to breast cancer metastases. One approach is to determine ways of selectively increasing strontium uptake at the metastatic site. Bisphosphonates are useful in the treatment of osteoporosis and bone metastases. Bisphosphonates sequester calcium and strontium. Strontium was non toxic to human breast cancer cells. Bisphosphonates are charged molecules that do not easily cross hydrophobic cell membranes. The nature of the counter ion to the anionic phosphonate was found to have an effect on the cellular uptake of bisphosphonate such as etidronate. The cytotoxicty of zoledronic acid towards MCF-7 cells greater than pamidronate and etidronate. The presence of strontium chloride in the culture medium inhibited calcium uptake by MDA-MB 231 cells. Etidronic acid treatment perturbed cell cycle progression of MCF-7 cells, causing a G2/M block and decreased the proportion of cells in S-phase. This suggests that a bisphosphonate such as etidronate may be useful in combination with ionizing radiation for treatment of bone metastases.

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Table of Contents

INTRODUCTION

In this project we have concentrated mainly on establishing monolayer cultures of human fetal osteoblasts, breast cancer cells and murine osteoclasts. We have also established the conditions of low calcium levels for tissue culture experiments. Experiments are in progress for estimating calcium and strontium in cells. Tasks 2, 4 and 5 have not been initiated, while progress has been made in tasks 1, 3, 6 and 7. One manuscript and four abstracts have been published during this report period.

BODY

BACKGROUND

More than 50% of patients with primary breast cancer will eventually develop bone metastases and 75% of patients with breast carcinoma were found to be with bone metastases at autopsy (1,2). In spite of skeletal metastases, breast cancer patients can survive for long periods (5 year survival is about 20%), but these persons will have much pain and suffering due to cancer mediated bone destruction (2). In addition to bone pain, these individuals may develop bone fractures, spinal cord compression (which can cause paralysis) and hypercalcemia of malignancy (HCM) (2-4). Breast neoplasms have a strong tendency to metastasize to the bone. For this to occur, both the tumor cell and host cells at the site of metastasis interact significantly and selectively (5-7). Once the cancer cell reaches the bone surface, the proteolytic enzymes of the neoplastic cell can facilitate the break down of the stroma and pave the way for metastasis to become entrenched. Another possibility is for the cancer cell to recruit and promote the proliferation of osteoblasts (bone forming cells) and new bone formation. Soluble growth factors from the cancer cell enable new bone to be formed around the neoplastic cell. Alternatively the growth factors produced by the migrating tumor cell could stimulate the proliferation of osteoclasts which are involved in bone destruction. The breast carcinoma cells can also destroy the bone. Destruction of the bone by the metastatic process can lead to hypercalcemia and associated problems. Certain breast tumors produce parathyroid hormone which promotes bone resorption. In particular, parathyroid hormone stimulates osteoclasts which degrade the bone. It appears that parathyroid hormone stimulates the growth of osteoclasts, and enhances parathyroid hormone production by the bone compared to normal tissues and tumor (7,8). Other factors known to increase osteoclast mediated bone resorption are interleukin 1 (9), interleukin 8 (10), interleukin 6 (11) and cathepsin K (12).

Preosteoblasts are differentiating intermitotic cells derived from vegetative intermitotic mesenchymal stem cell. The preosteoblasts differentiate further to yield mature osteoblasts that are involved in bone matrix synthesis. Preosteoclasts are also of the differentiating intermitotic types that are derived from hematopoietic stem cell. Preosteoclasts differentiate further into mature multinucleated osteoclasts that are involved in bone resorption. Cytokines such as TGF-β, macrophage-colony stimulating factor (M-CSF), IL-6, TNF-α, and osteoprotogerin (OPG)/osteoprotogerin ligand (OPG-L) from a variety of sources including mature osteoblasts are involved in promoting proliferation and differentiation of preosteoclasts to mature osteoclasts (13). Estrogen deficiency is associated with enhanced bone osteoclast formation and bone loss. Correction of estrogen deficiency prevents bone loss. Estrogen action is mediated through cytokine production. Bisphosphonates also protect against bone loss. There is evidence to suggest that both estrogen and bisphosphonates can have direct effects on osteoblasts and osteoclasts (13-15). There

are examples of bisphosphonates covalently linked to estrogens and anti-estrogens (16, 17). The anti-estrogen, tamoxifen and selective estrogen response modifiers protect against osteoclast mediated bone resorption (18, 19). The effects of conjugates of bisphosphonates with estrogens and anti-estrogens on osteoblasts and osteoclasts are not known. Investigation of such conjugates may help identify compounds that protect against bone lysis caused by breast cancer metastases.

The radioisotope Strontium-89 (Sr-89) has a physical half life of 50.5 days. It is a pure beta emitter (undergoes beta minus decay with a beta emission of 1.463 MeV). The maximum range of the beta emission is about 8 mm. Strontium is a calcium mimic and a bone seeking mineral. When in the bone the radiation from this isotope is mainly to the cortical and trabecular bone, with less to bone marrow and minimal dose to adjacent soft tissues. Strontium-89 is usually given at a dose of 4 mCi (or 40-60μCi/kg body weight [1.5MBq/kg] is used as a single injection. About 65% of the patients gain relief from pain. The success of Sr-89 treatment depends on the ability of the radioisotope to infiltrate into the osteoblasts and osteoclasts, Once the radioisxzotope is taken up by the cells, the beta emission can damage the cell. If the isotope is retained long enough then the accumulated damage can be lethal to bone cells. Strontium-89 has been used for palliative treatment of bone metastases from breast and other cancers (20-24). Strontium-89 is sold under the trade name metastron. We have used metastron for treating bone pain in about 41 patients (25). There is considerable variation in treatment response with respect to pain relief. There may be several reasons for this. For this treatment to be effective, radioactive strontium has to be taken up in sufficient amounts by the intended target (osteoblasts and or osteoclasts). Sr-89 does not exert an appreciable lethal effect towards tumor cells. Since strontium is a calcium analog, the uptake of strontium may depend on a variety of factors including systemic calcium levels. If the systemic calcium levels are high, cellular uptake of strontium may be poor because calcium may competitively inhibit the uptake of strontium.

Bisphosphonates are useful in the treatment of bony metastases, especially those traceable to an osteoclast etiology (26-34, 35-37). Bisphosphonates and estrogen metabolites appear to have a direct effect on bone cells (38, 39). Bisphosphonates also appear to have antineoplastic effect by inducing apoptosis of tumor cells *in vivo* and *in vitro* (40). Osteoprotegerin (OPG) is a potent inhibitor of osteoclast formation and activity. OPG is a decoy receptor which neutralizes OPG-ligand (OPG-L) which is the ultimate effector of osteoclastogenesis (41).

Recently there has been some concern about adverse effects of bisphosphonates such as the development of osteonecrosis. These effects are usually seen with prolonged use of bisphosphonates. The severity of the adverse effects appears to be related to the potency of bisphosphonates and instances of osteonecrosis appear to be more common with newer generation bisphosphonate such as zoledronic acid compared to earlier generation bisphosphonates (44, 45)..

HYPOTHESIS/RATIONALE/PURPOSE

The purpose is to improve the use of Sr-89 and bisphosphonates for treating bone metastases from breast cancer. The rationale is as follows: Nearly 50% of breast cancer patients develop bone metastases due to osteotropic nature of the primary breast cancer cells. Breast cancer cells, osteoblasts and osteoclasts have some growth factors in common. Thus the breast cancer cells promote the growth of either or both types of cells. The osteoblsts surround the cancer cell which has adhered to the bone matrix and starts depositing new bone which ultimtely leads to bony

metastasis. In contrast, the osteoclasts utilize the growth factors differently. They destroy the bone by resorption which results in hypercalcemia.

Sr-89 is usually satisfactory for the treatment of lesions caused by osteoblasts. Osteoblastic activity attracts breast cancer cells and utilizes calcium for making new bone. Strontium is an analog of calcium. Sr-89 is easily taken up instead of calcium by the active osteoblasts. Once inside the osteoblast, the beta emission from the radionuclide inactivates the osteoblast. This can lead to some relief from pain, If the lesion is caused by osteoclast, which does not consume calcium or strontium ions avidly, Sr-89 is not quite so effective against osteoclasts. Bisphosphonates are pyrophosphate analogs which have a rather high affinity for bone. Although bisphosphonates such as pamidronate are useful in the treatment of bony metastases, it is important to know if the high levels of hypercalcemia have to be decreased in order to achieve effective treatment with pamidronate or Sr-89. Since bisphosphonates and Sr-89 complement each other, addition of a bisphosphonate can enhance the efficacy of Sr-89 and vice versa. Such logic can be extended to arrive at other combinations based on our knowledge of the mechanisms involved.

Hypothesis 1: Calcium concentration in the system affects strontium uptake by the cells. For improved use of Sr-89, strategies for decreasing cellular calcium pools are necessary. Gallium nitrate or a bisphosphonate can be used for lowering the calcium levels in cells.

Hypothesis 2: Antiestrogens and estrogens can modify the effects of bisphosphonates on osteoblasts and osteoclasts. Bisphosphonates linked covalently to anti estrogen and estrogen moieties will have greater affinity for bone cells and may be more active.

Hypothesis 3: Hormones and hormone antagonists can be combined for modulating the effects bisphosphonates on bone cells.

Specific Aims:

1. Treat osteoblast and osteoclast-like cells in culture with graded concentrations of strontium in the presence of different amounts of calcium and measure the kinetics of uptake and retention of strontium by cells. Atomic absorption spectroscopy will be used for estimating strontium concentration.
2. Test the influence of the bisphosphonates pamidronate and its more potent analog zolendronate on the uptake of strontium by the osteoblast cell lines in culture. It is not known if bisphosphonates alter the bioavailability of strontium ions.
3. Synthesize bisphosphonates conjugated to estrogen and anti-estrogen moieties and test their effects on the viability of breast cancer cell lines and human fetal osteoblasts in culture. Cell viability will be assessed on the basis of apoptosis assays and clonogenicity measurements where feasible.
4. Determine if gallium nitrate, calcium channel blockers, nifedipine and verapamil and the antiestrogens tamoxifen and raloxifene alter strontium uptake by human fetal osteoblasts. grown as monolayers and as multicell spheroids.

Both estrogen receptor positive and receptor negative human fetal osteoblasts and breast cancer cells are being utilized in our experiments. Estrogen receptor positive human fetal osteoblast (hFOB/ER9) and estrogen receptor negative human fetal osteoblasts (hFOB1.19) are to be studied. Estrogen responsive MCF-7 and estrogen independent MDA-MB231 are some of the breast cancer cells in our laboratory.

STATEMENT OF WORK

Task 1. Establish osteoclast and osteoblast cultures (months 1 through 6). Standardize assays for strontium and calcium using atomic absorption spectrophotometry (months 1 through 3). Determine the baseline values of strontium and calcium in cultures grown in defined media. Evaluate the effect of calcium levels in the medium on strontium uptake by hFOB1.19 and hFOB ER/9. Compare the results obtained using serum-free and serum supplemented culture media. Test the effects of gallium nitrate and calcium channel blockers verapamil and nifedipine on strontium uptake and retention (months 1 through 15).

Task 2. Synthesize bisphosphonates conjugated to estrogenic and antiestrogenic moieties (months 1 through 18).

Task 3. Test the effects of bisphosphonates (including pamidronate, zolendronate and those synthesized in task 2) on strontium uptake by osteoblasts (months 6 through 30).

Task 4. Determine the influence of gallium nitrate on the uptake of strontium by the afore-mentioned cell lines (months 2 through 12).

Task 5. Measure the uptake and retention of strontium by multicellular spheroids of osteoblasts in the presence and absence of agents which affect calcium homeostasis (months 6 through 30).

Task 6. Evaluate the differences, if any, in the uptake kinetics of strontium and calcium in the different cell lines. Determine any correlations that may exist among the cellular levels of calcium and strontium on the cytotoxicity of bisphosphonate. Utilize isobologram analysis to reveal any synergistic or antagonistic interactions between bisphosphonates and strontium and/or calcium (throughout the 36 month project period).

Task 7. Assays for strontium, calcium and clonogenicity assays for cell viability and apoptosis will be carried out throughout the 36 month project.

Materials and Methods:

Cell lines: Estrogen responsive MCF-7 and estrogen independent MCF-7MDR clone 10.3 and MDA-MB231-luc human breast cancer cell lines were maintained as monolayer cultures growing in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine, pyruvate, insulin,

penicillin and streptomycin. MCF-7 cells were also grown as multicellular spheroids. We have also grown spheroids with matrigel.

Culturing hFOB 1.19 and hFOB/ER cell lines. These osteoblast cells (42, 43) were obtained from Dr. Thomas Spelsberg of Mayo Clinic, Rochester. The following is their description of the cells along with the recommended procedure for the growth and maintenance of these cells.

The hFOB 1.19 cells were cultured as monolayers at 34^{o} C in DMEM-F12 medium supplemented with 10% fetal bovine serum and 300μg/ml geneticin, and induced to differentiate more fully either by culturing past confluence or by culturing at 39^{o} C, as described by Harris SA et al, Bone Miner Res 10:178-186, 1995 (42).

The hFOB/ER9 cell lines were derived from the hFOB 1.19 cells were also cultured at 34^{o} C in DMEM: F12 (1:1), but supplemented with 10% charcoal-stripped FBS and either geneticin at 300μg/ml or hygromycin B at 100μg /ml.

Spheroid culture: We have acquired high aspect ratio vessels for culturing hFOB spheroids. These spheroids are more difficult to grow compared to multicell spheroids of MCF-7 cells. MCF-7 cells form spheroids in stationary cultures with unstirred medium and in spinner flasks. We have utilized matrigel to facilitate spheroid formation from MCF-7 cells in stationary cultures.

The breast cancer line MCF-7 cells were obtained from ATCC and grown in RPMI 1640 medium. Pure cell cultures were incubated at 37 ^{0}C for 6 days. On the third day, media was removed and fresh media added to maintain good cell growth. At the end of 6 days, media was removed and the adherent cells recovered by adding Trypsin/EDTA. The trypsinized cell culture was collected in fresh media and 2 x 10 ^{6} cells/ml used for treating the cells. Cells were seeded on plates and incubated overnight. The following nine treatments were imposed on the cell cultures and incubated for 6 and 20 hours. Control; 0, 3.5 mM and 7.0 mM Strontium Chloride applied separately and in combination with 10 and 20 mM etidronic acid. At the end of 6 and 20 hours incubation, cells were harvested by scraping and transferred into 15 ml centrifuge tubes. One set was used for cell cycle analysis and the second set was reserved for DNA extraction.

MTT Assay for Cell Viability:

The cells were treated with graded concentrations of etidronate for different treatment periods of incubation (1, 3, 6 and 12 hrs). Mitochondial dehydrogenase levels, which are correlated to cell viability, were determined by the enzyme mediated cleaving of the tetrazolium salt ring of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide to yield purple formazan crystals. These crystals were dissolved in isopropanol, and the absorption at 560 nm was determined spectrophotometrically. Appropriate control experiments were run to determine spectrophotometric background and absorption due to reagent blanks.

Clonogenicity Assay:

Following treatment, cells were trypsinized at 37°C for 5 minutes, and pipetted up and down 5 times to break up cell clumps and obtain a single cell suspension. The same volume of single cell suspension was plated on 100-mm tissue culture dishes with fresh medium and kept at 37°C, 5%

CO_2 incubator for 10~14 days. Colonies consisting of more than 50 cells were counted. Survival was calculated as percentage rate of the number of colonies formed at a given treatment condition to the number of colonies produced by related untreated control cells.

Hyperthermia treatment:

For 96-well plate: Cells were seeded in sterile 96-well plates at a density of 1×10^4 cells/well and incubated overnight. For 24-well plate: cells were seeded at densities of 1×10^3, 1×10^4, 1×10^5 and 5×10^5 /well. Hyperthermia was applied at 43°C by sealing the plates with parafilm and enclosing in a Ziploc bag and then immersing the bag into a temperature controlled water bath maintained at $43^o(\pm 0.1°C)$. The continuous heating period ranged from 10 to 120 minutes. Controls were sealed in ziplock bags and immersed in a 37°C water bath. After heating, plates were ready for optical imaging, and for MTT and clonogenic assays.

Apopain Assay:

Apopain/Caspase 3 is derived from the proenzyme CPP32 at the onset of apoptosis and plays a pivotal role in programmed cell death. This assay indicates apopain activity associated with apoptosis. The FluorAce apopain assay kit was used (Bio-Rad, Catalog number 170-3130).

Flow Cytometry Analysis for Cell Cycle and Apoptosis:

The effect of etidronate treatment on cell cycle and apoptosis was analyzed using flow cytometry assay. The cells were trypsinzed and washed twice with PBS after treatment. The suspended cells were fixed with 80% ethanol for 30 min on ice, and then centrifuged for 5 min at 1500rpm. The fixed cells were washed again with PBS and the supernatant was removed. The cells were stained at 4^o C in the dark with 1 ml of propidium iodide (PI) solution and/or fluorescein-conjugated annexin V (apoptotic marker), and then stored at 4^oC until analysis. The cell cycle distribution was analyzed by FACS caliber flow cytometry (Becton Dickson, San Jose, CA). Ten thousand cells were analyzed per sample. PI solution contained 100 Units/ml or 50 μg/ml RNase A and 50 μg/ml PI in PBS. Similarly the effect of different concentrations of strontium chloride and etidronate on cell cycle was analyzed using flow cytometry.

Cytotoxicity of Etidronic Acid Towards Human Breast Cancer Cells.

Human breast cancer frequently metastasizes to the skeleton to cause osteolysis and subsequent pain, pathological fracture, and hypercalcemia. The bone continuously releases growth factors stored in bone matrix by bone resorption and provides a favorable environment for metastatic breast cancer cells to proliferate. Reducing the amount of bone destruction and increasing the rate of bone repair can reduce pain and lower the risk of fractures by keeping bones strong. Inhibition of bone resorption is a useful adjuvant therapy in patients with breast cancer. Bisphosphonates are potent inhibitors of osteoclastic resorption and are used in the treatment of osteoporosis, hypercalcemia, and bone metastases. Alendronate, etidronate and risedronate increase bone density and prevent spine and hip fractures (46). Etidronic acid has been shown to inhibit bone resorption and increase bone mineral density.These drugs bind permanently to the surfaces of the bones and slow down the osteoclasts (bone-eroding cells). This allows the osteoblasts (bone-building cells) to work more effectively. Bisphosphonates may induce human osteoblast differentiation via inhibition of the

mevalonate pathway or other mechanisms. These drugs may also induce cell death by apoptosis through the inhibition of protein farmesylation and geranylgeranylation. Cell proliferation of normal and cancer tissues requires post-translational isoprenylation by farmesyl-transferase and geranylgeranyl-transferase.

Strontium, an isotope that imitates the in-vivo behavior of calcium, is known to stimulate bone formation and has been used in the treatment of osteoporosis. It is readily taken up by bone and is concentrated at the osteoblastic skeletal metastatic sites. The combined effects of etidronate and strontium on breast cancer cells has not been studied.. The purpose of the present investigation is to determine the combined effects of etidronate and strontium on apoptosis and the mutational status of the p53 gene in MCF-7. The tumor suppressor gene p53 plays an essential role in cell proliferation and apoptosis. Missense mutations of p53 occur in 50 % of all cancers. Due to its relevance to cancer therapy, most studies have focused on the cellular consequences of p53 activation in relation to cytotoxic drugs. Anticancer drugs are known to induce apoptosis by triggering biochemical events involved in apoptotic pathways and in cell cycle regulators (47, 48). Results from this study will provide some insight into the management of bone metastases in breast cancer patients.

Etidronic acid (1-hydroxyethane-1,1,-diphosphonic acid; 1-hydroxyethylidenediphosphonic acid) (Eti) affects calcium metabolism and slows down abnormal bone resorption. The anti osteolytic activity of this compound has led to research on the use of bisphosphonates for the treatment of Paget's disease, osteoporosis and cancer metastases to the bone. Strontium, which imitates the in vivo behavior of calcium, stimulates bone formation and has been used in the treatment of post menopausal osteoporosis. There have been several reports on anti tumor effects of bisphosphonates. The cytotoxicity of etidronic acid (a first generation bisphosphonate) towards MCF-7 human breast cancer cells and its multidrug resistant derivative MCF-7 clone 10.3 cells, was studied in the presence and absence of strontium chloride (Sr). Clonogenicity assays revealed that a 24 hour exposure to etidronic acid (10 mM) was more toxic to MCF-7 compared to MCF-7 clone 10.3 cells, while the addition of strontium chloride had no effect. Flow cytometry studies revealed that etidronic acid caused a decrease in the s-phase population with concomitant increase in G2/M phase population . Again strontium was without any effect. Within the cell cycle, late s-phase cells are the most radioresistant while G2/M cells are the most radiosensitive. Therefore the decrease in s-phase population with corresponding increase in G2/M would position the cells in a relatively more radiosensitive setting. Such a shift in cell cycle distribution may be useful if etidronic acid were combined with radioactive strontium (^{89}Sr, metastron), which is a beta emitter used in the treatment of bone metastases from breast cancer.

Inhibition of cellular uptake of calcium by strontium.

Our assumption that calcium levels would influence cellular uptake of strontium were not borne out in hormone independent MDA MB-231 cells. In fact the presence of strontium in the medium inhibited calcium uptake by cells. Preliminary indications are that strontium in combination with the calcium channel blocker verapamil strongly inhibited calcium uptake.

The normal steady state calcium levels in MDA-MB231 cells was around 230 nM based on digitized fluorescence microscopy of single cells using Fura-2, AM ester as a fluorescent probe. Even a 10 minute exposure to strontium chloride (1 mM) decreased cellular calcium levels to 140 nM. More experiments are needed to understand the uptake kinetics of strontium and calcium in cells.

Comparison of cell viability assays,

We observed that cells deemed to be viable on the basis of trypan blue dye exclusion as well as the MTT assay proved to be dead on the basis of clonogenicity assays. Since clonogenicity is the gold standard for evaluating cancer chemotherapeutic agents, we compared different assays for cell viability.

KEY RESEARCH ACCOMPLISHMENTS

- Monolayer cultures of the cell lines needed for our research have been established, but only MCF-7 human breast cancer cells have been grown consistently as multicell spheroids. Spheroid formation was facilitated in the presence of matrigel.

- Assays for cell viability have been standardized and utilized for evaluating the cytotoxicity of etidronate and analogs towards the different cell lines in culture.

- Presence of strontium in the culture medium decreases calcium uptake by hormone independent breast cancer cells.

- Toxicity of etidronate towards osteoclast and breast cancer cells was demonstrated. The cytotoxicity of etidronic acid was enhanced by hyperthermia.

- Flow cytometry assays for cell cycle analysis and estimation of apoptosis have been standardized and applied to study the effect of etidronate towards breast cancer cells.

- Strontium chloride (up to 7 mM) was non toxic to breast cancer cells. Strontium decreased the uptake of calcium by MDA-MB-231 breast cancer cells.

REPORTABLE OUTCOMES

PUBLICATIONS

1. Sridhar, R. RNA processing in health and disease: a brief review. J Clin Ligand Assay 28 (#2): 61-67, 2005

ABSTRACTS

1. Sridhar R, Kassa A and Ashayeri E. Modulation of bisphosphonate toxicity towards human breast cancer cells in culture. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Abstract P43-17, Philadelphia, PA, 2005. (Abstract)

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4. Zhang R, Zhou Y, Kassa A, Gu X, Ashayeri E and Sridhar R. *In Vitro* Cytotoxicity of Lipoxygenase Inhibitors towards PC3 and DU-145 Human Prostate Cancer cells, RCMI International Symposium on Health Disparities: Scientific Program and Abstracts, pp 73-74, Baltimore, MD, December 8-11, 2004.

CONCLUSIONS

In addition to their effect on calcium, bisphosphonates are cytotoxic towards breast cancer cells in culture.

The bisphosphonate etidronate is toxic to breast cancer cells and perturbs the cell cycle as judged by flow cytometry. Combining etidronate with calcium or strontium chloride causes precipitation of insoluble material. Calcium and strontium salts of etidronic acid are sparingly soluble.

Strontium chloride up to 7 mM was not toxic to MCF 7 breast cancer cells in culture. However, strontium chloride caused the precipitation of etidronic acid. Presence of strontium in the culture medium decreases calcium load in breast cancer cells.

Bisphosphonates decrease the proportion of cancer cells in the DNA synthetic phase (s-phase). This may be due to the replacement of critical pyrophosphate links by non hydrolysable phosphonate linkage to bases in nucleotides. We have obtained ^{31}P NMR spectrum of etidronic acid. This will be useful for monitoring its presence in cell cultures.

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List of appended abstracts and paper

Abstracts:

1. Sridhar R, Kassa A and Ashayeri E. Modulation of bisphosphonate toxicity towards human breast cancer cells in culture. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Abstract P43-17, Philadelphia, PA, 2005.

4. Zhou Y, Pang X, Zhang R, Gu X, P Kc, Sridhar R. Loperamide sensitizes multidrug resistant MCF-7 clone 10.3 human breast cancer cells to doxorubicin, Proc. Amer. Assoc Cancer Res. 46: 538, 2005.

5. Zhang R, Brown S, Guerrier K, Kassa A,Zhou Y, Gu X, Ashayeri E and Sridhar, R. Cytotoxicity of Lipoxygenase Inhibitors towards prostate cancer cells in culture, Proc.Amer. Assoc. Cancer Res. 46: 5863, 2005.

7. Zhou, Y., Sridhar, R., Gu, X., Pang, X., Balachandran, R., Magarian, R.A., and Day, B.W. Evaluation of a series of 1,1-dichloro-2,3-di-and tri-arylcyclopropanes as MDR reversing agents. Proc. Amer. Assoc. Cancer Res. 45: Abstract 2139, 2004

8. Zhang R, Zhou Y, Kassa A, Gu X, Ashayeri E and Sridhar R. *In Vitro* Cytotoxicity of Lipoxygenase Inhibitors towards PC3 and DU-145 Human Prostate Cancer cells, RCMI International Symposium on Health Disparities: Scientific Program and Abstracts, pp 73-74, Baltimore, MD, December 8-11, 2004.

Paper

1. Sridhar, R. RNA processing in health and disease: a brief review. J Clin Ligand Assay 28 (#2): 61-67, 2005.

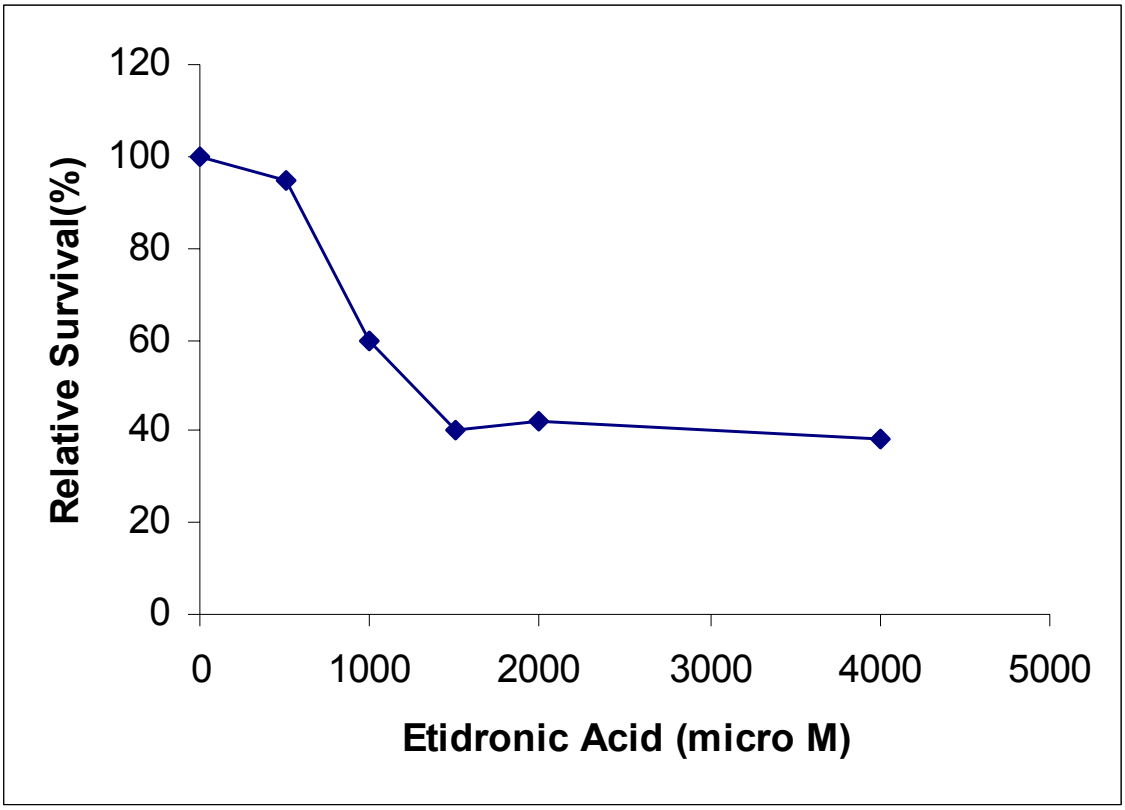
Appendix 1: Cytotoxicity and cell cycle effects of bisphosphonates:

Bisphosphonic acids are relatively strong acids, which are charged polar compounds. Because of their charged nature, these compounds do not easily cross hydrophobic cell membranes. The acidic groups in bisphosphonic acids containing one or more basic nitrogen atoms are more potent pharmacological agents compared to those without a basic nitrogen atom in their structure. It is possible that the basic nitrogen atom ties up the acidic phosphonic acid by an intramolecular or intermolecular interaction. Where possible an intramolecular unimolecular reaction will be favoured over intermolecular interaction that will obey bimolecular kinetics. The interaction of the bisphosphonic acid group with the basic nitrogen atom will result in a species that is likely to cross the cell membrane more easily than bisphosphonic acid derivatives lacking a basic nitrogen atom.
Formation of bisphosphonate salts with divalent ions such as calcium or zinc may also increase cellular bioavailability of bisphosphonates. Experiments utilizing chelators such as EDTA or EGTA are sometimes used to rule out calcium mediated effects on cytotoxic effects of bisphosphonates. These experiments ignore the possibility that insoluble or slightly soluble calcium bisphosphonates can be involved in the mechanism of bisphosphonate action. Bisphosphonates, upon entering the cell may become trapped as insoluble calcium salt. On the other hand if the calcium bisphosphonate is formed outside the cell it will not be bioavailable at the cellular level. This may explain the high residence time of bisphosphonates which may find their way to the site of bone formation or bone metastases. Zoledronic acid is a highly potent bisphosphonate, which is in clinical use for the treatment of osteoporosis and bone metastases. It is significant that the imidazole moiety in its structure provides two basic nitrogen atoms for neutralizing the bisphosphonic acid moiety. This internal neutralization of the net acidity may partially account for its high potency.

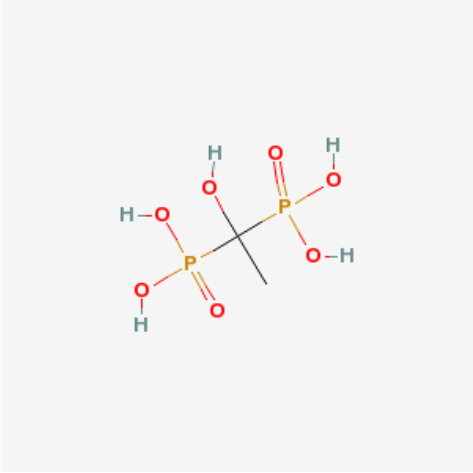
In principle, neutralizing the bisphosphonic acid moiety either through the use of divalent cations or organic amines should improve the cross membrane transport of bisphosphonates into cells. Liposome encapsulated bisphosphonates are more cytotoxic than the corresponding unencapsulated free drugs since the liposomal formulation permits improved delivery of bisphosphonates to cells.

Some polyamines marginally increased the cytotoxicity of etidronic acid when tested in the millimolar range. The results varied from experiment to experiment and even a slight protection was seen when imidazole was combined with etidronate. Imidazole interacts with etidronic acid to neutralize the acidity. When calcium chloride solution was added to the neutralized solution, a milky solution was formed, presumably due to the insolubility of calcium etidronate. Photomicrograph of the precipitate is shown. The formation of this insoluble product may account for the erratic kinetics of cell killing in the presence of bisphosphonates. Increasing the time of exposure had less than expected effect. Since bisphosphonates are not particularly unstable, we suggest

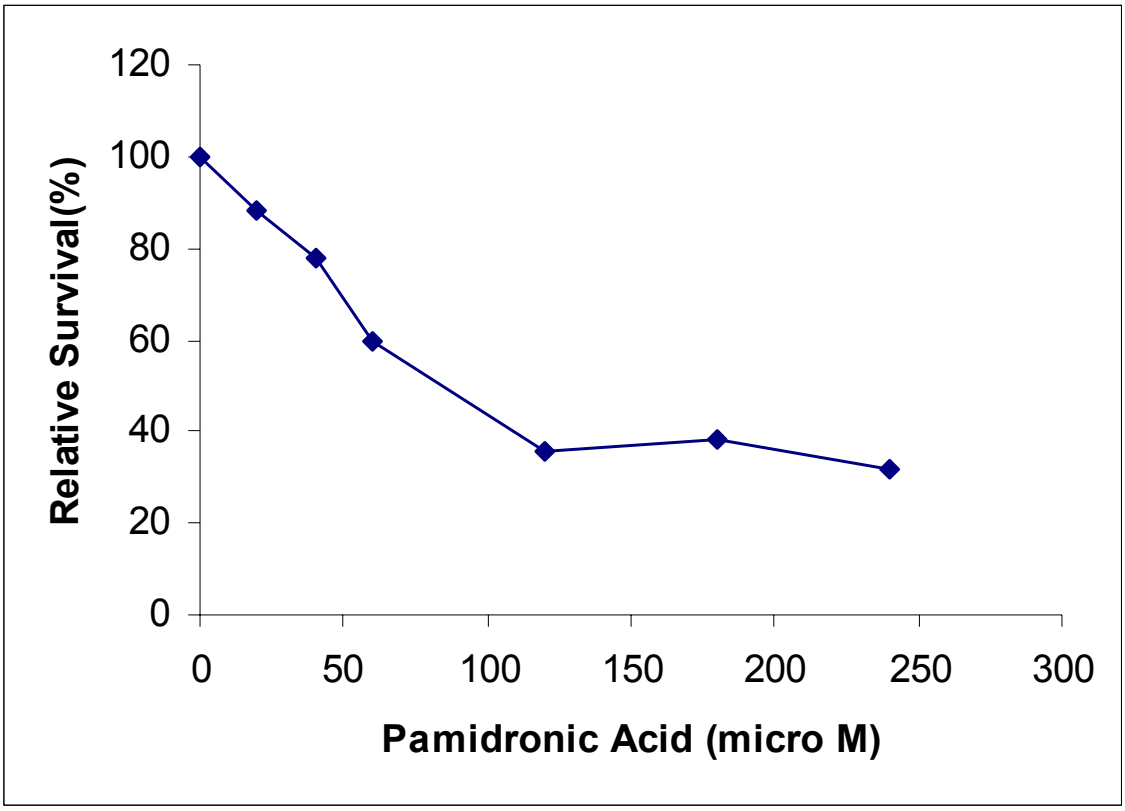
that their precipitation in the extracellular space renders them unavailable to most cells, except macrophages and other phagocytosing cells.



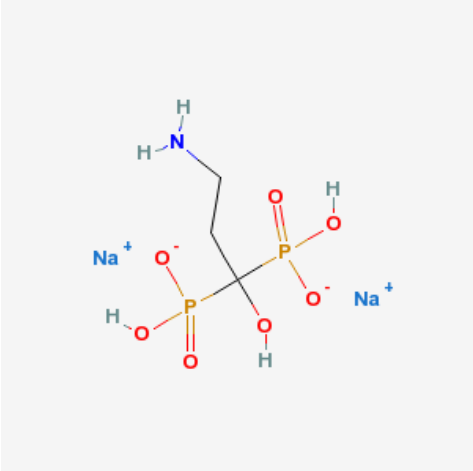
Cytotoxicity of etidronic acid towards MCF-7 cells



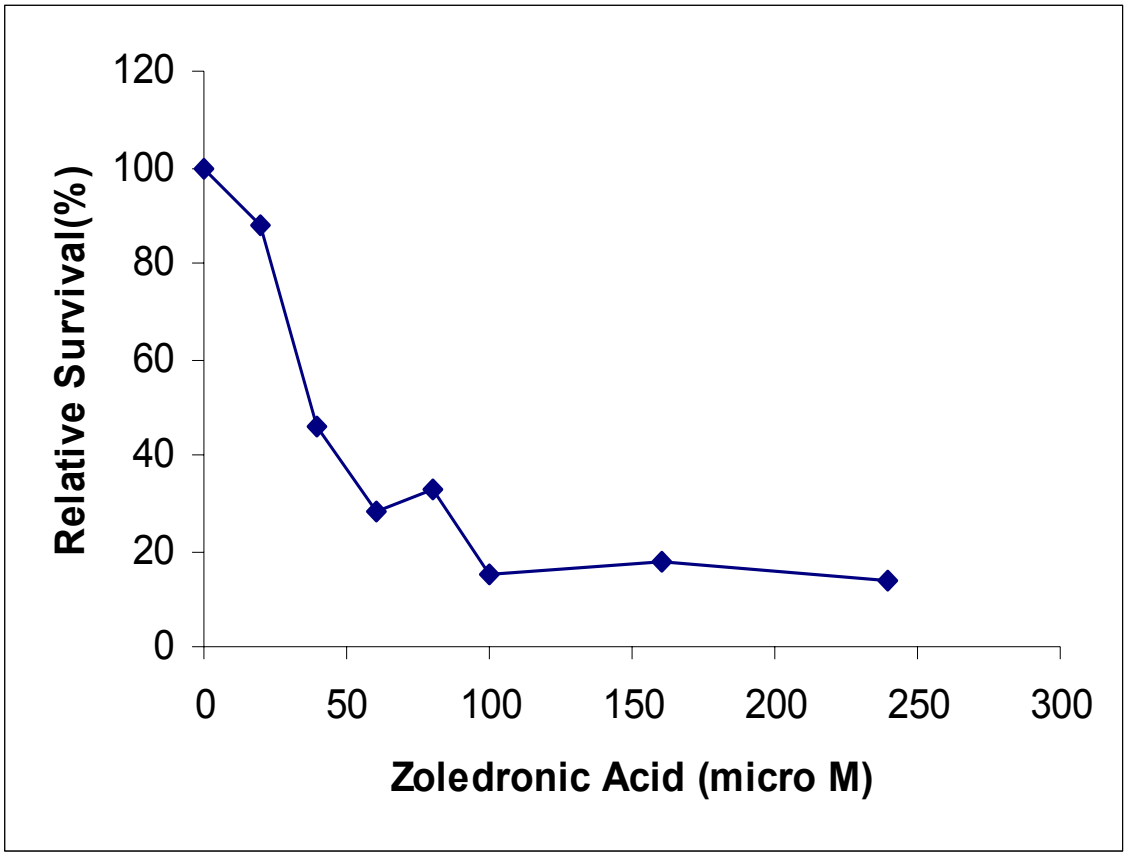
Structure of etidronic acid (Didronel)



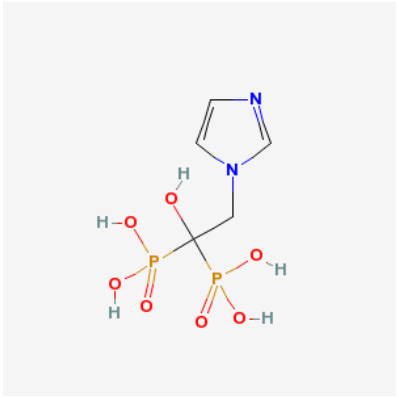
Cytotoxicity of pamidronic acid towards MCF-7 cells



Structure of pamidronate (Aredia)



Cytotoxicity of zoledronic acid towards MCF-7 cells

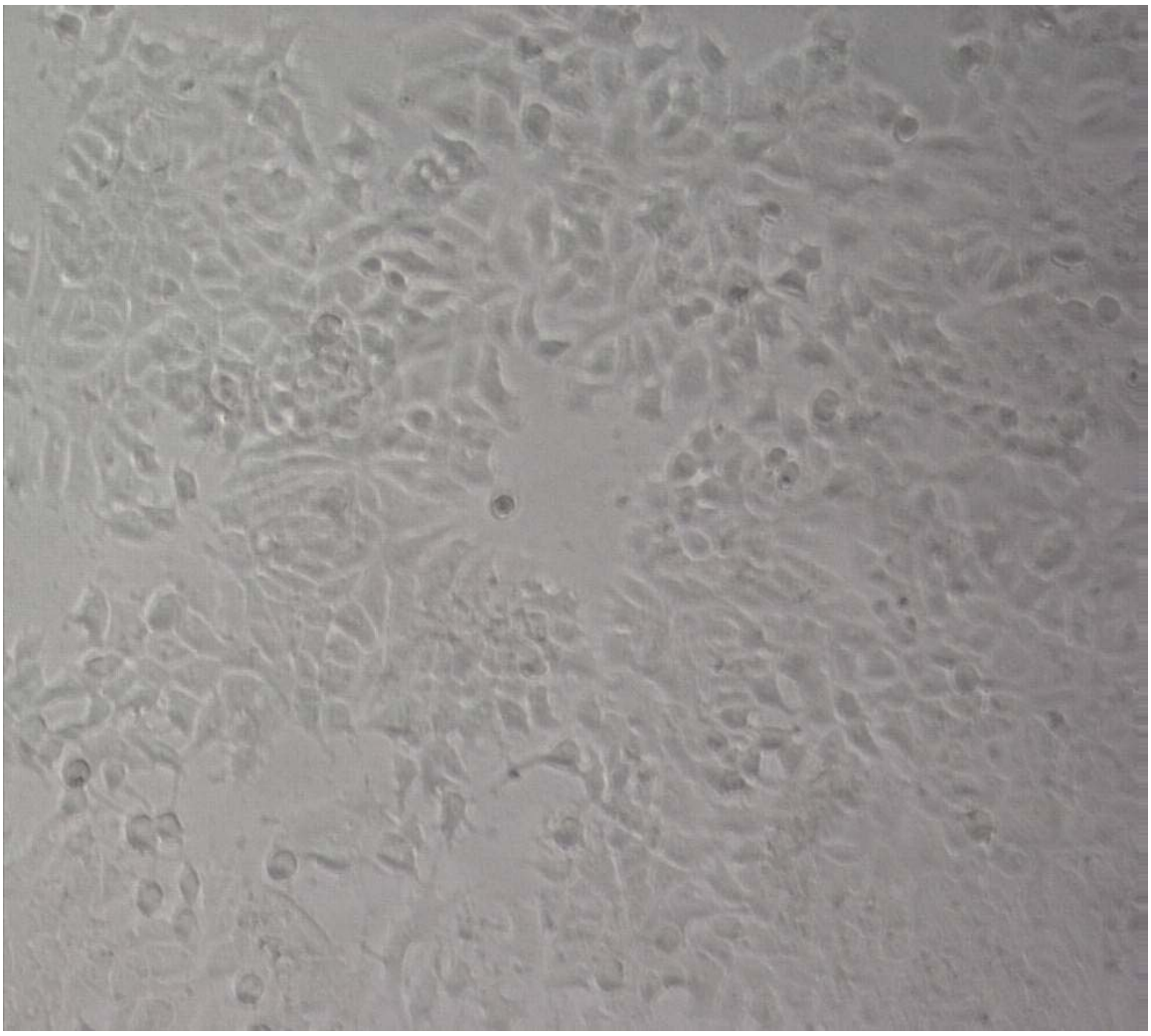


Structure of zoledronic acid (zometa)

Photomicrograhs: Light microscopy revealed damage caused to cultures exposed to etidronic acid. (Photomicrograph shown for control and treated cells are shown on page 26 and 27 respectively)

Addition of calcium chloride to etidronic acid caused precipitation of calcium etidronate, which could be observed under the microscope. (Photomicrograph shown) Bisphosphonates etidronate, pamidronate and zoledronate are cytotoxic to MCF-7 human breast cancer cells in culture, zoledronate being the most potent, followed by pamidronate and etidronate, which is the least potent. Prolonged use of the more potent bisphosphonate such as zoledronate (zometa) can cause the adverse effect of osteonecrosis in some patients. Etidronic acid is less potent but safer than zoledronic acid.

The dose response curves for cell survival for the three bisphosphonates indicated increased cytotoxicity with increasing dose. Surprisingly, increasing the duration of exposure to the bisphosphonates did not cause a proportional increase in cell killing. The cellular bioavailability of the polar bisphosphonates depends on the structure of the bisphosphonates. The highly charged bisphosphonate such as etidronic acid is not likely to be easily transported across cell membranes. Transport across cell membrane will be facilitated by masking the highly acidic bisphosphonate groups by cationic species.



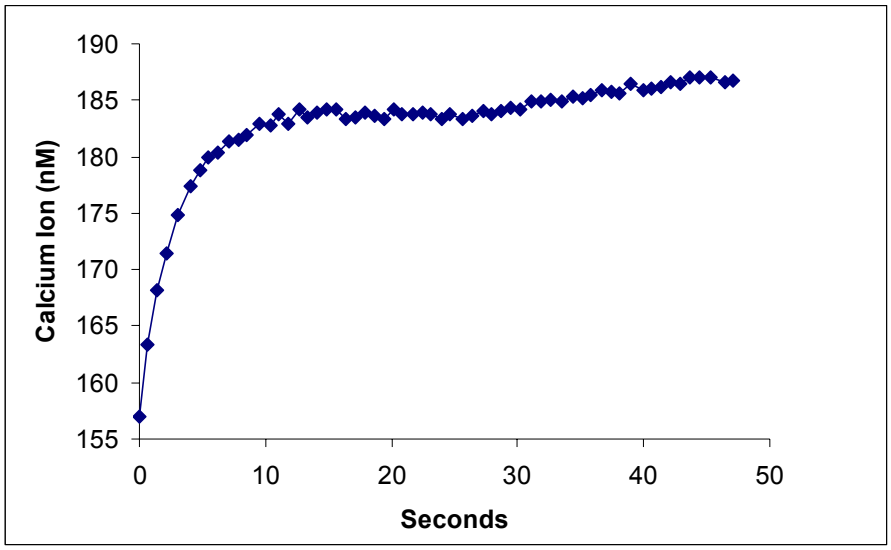
Photomicrograph of MCF control cells

Photomicrograph of etidronate treated cells

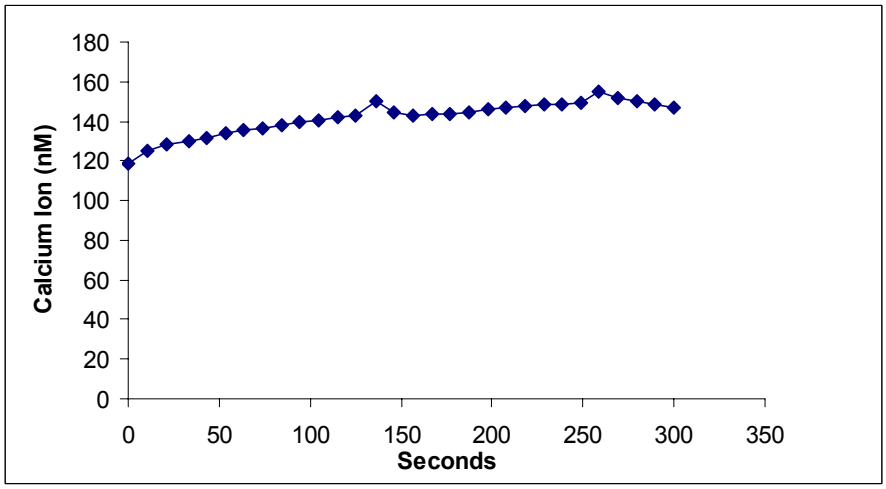
Photomicrograph of calcium etidronate precipitate

Appendix 2: Strontium inhibits cellular uptake of calcium

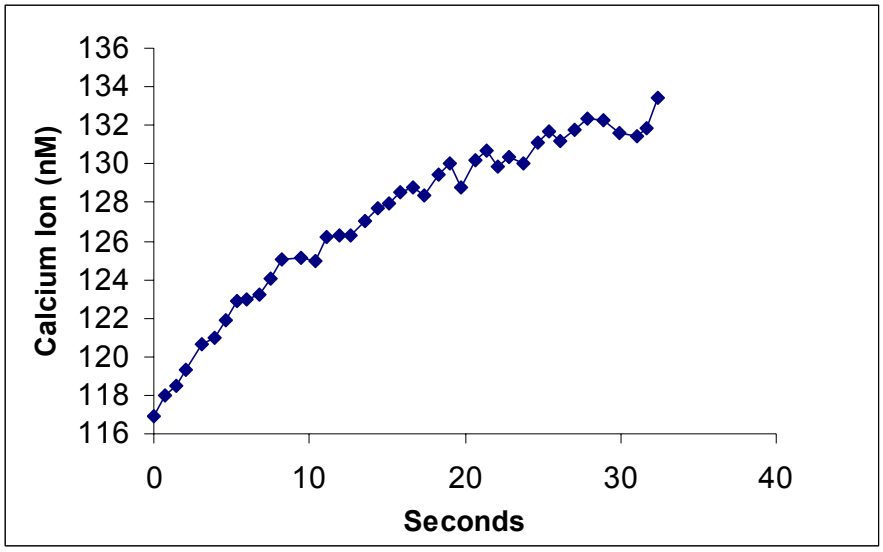
The effect of strontium chloride on intracellular calcium uptake from medium containing calcium and strontium, was measured using digitized fluorescence microscopy in cells preloaded with the fluorescent probe Fura-2, AM ester.



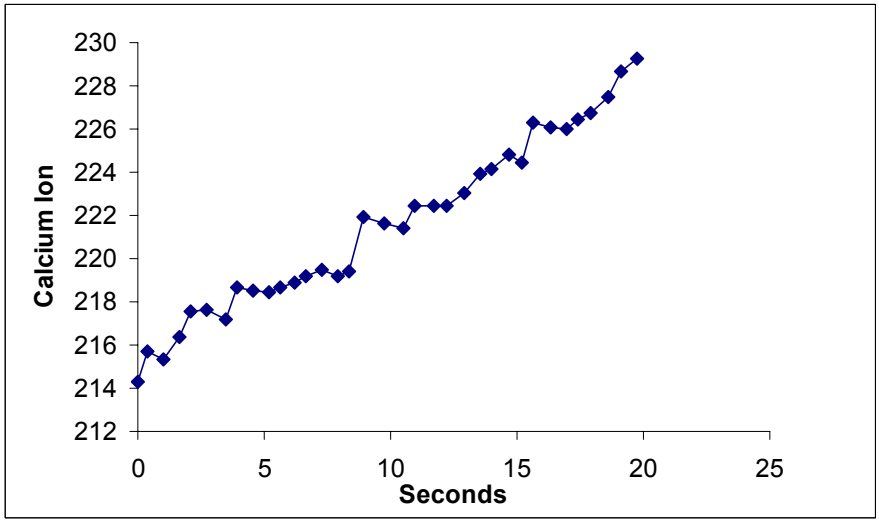
Intracellular calcium ion levels in MDA-MB231 human breast cancer cells incubated with strontium chloride for 30 minutes (average of 6 regions)



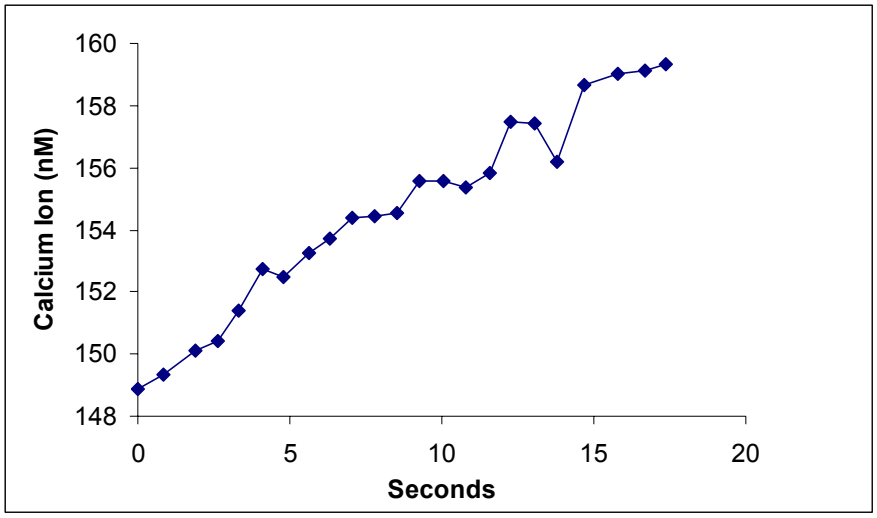
Intracellular calcium ion levels in MDA-MB231 cells incubated with 1 mM strontium chloride for 10 minutes (average of 6 regions)



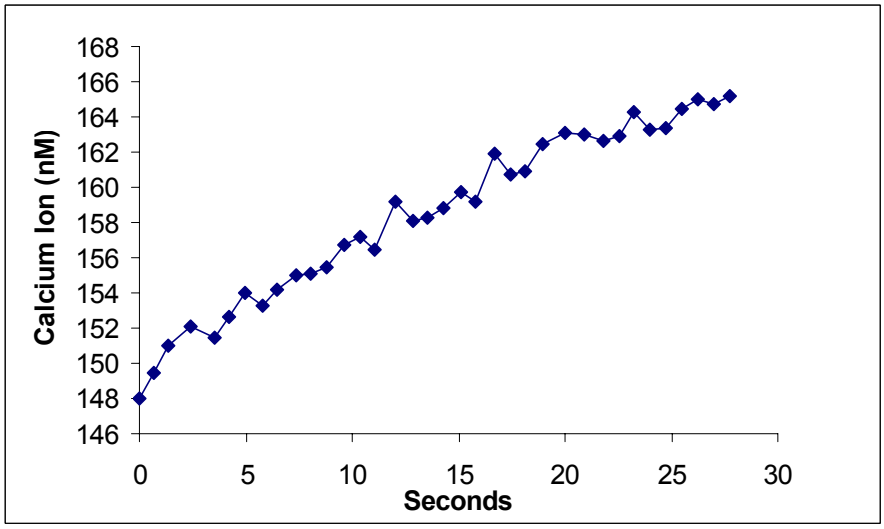
Intracellular calcium in MDA-MB231 cells incubated with sstrontium chloride (1 mM) for 15 minutes (average of 2 regions)



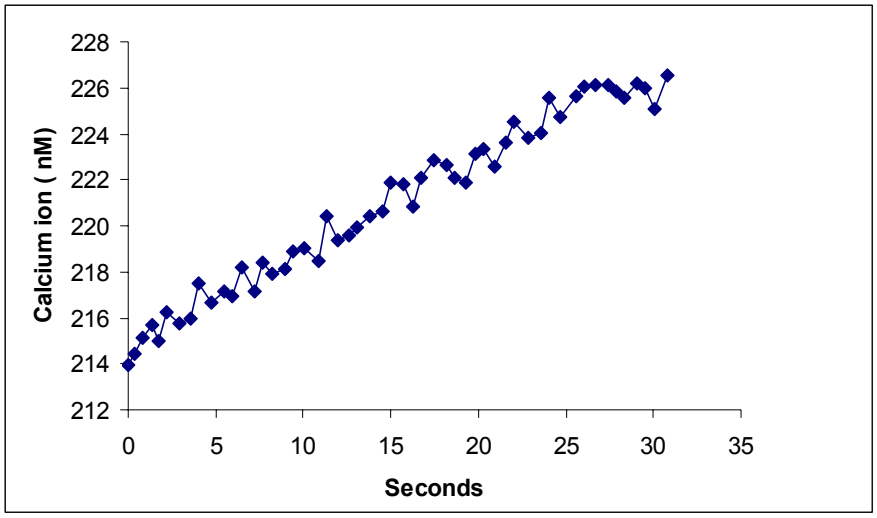
Intracellular calcium in MDA-MB231 control cells (average of 5 regions)



Intracellular calcium of MDA-MB231 cells incubated with strontium chloride (0.1 mM) for 10 minutes.



Intracellular calcium ion concentration in MDA-MB 231 cells incubated with strontium chloride (0.1 mM) for 15 minutes.



Intracellular calcium ion concentration in control MDA-MB231 cells incubated in medium without strontium chloride

Appendix 3: Comparison of cell viability assays.

Materials and methods

Cell line

MDA-MB-231-luc human breast cancer cell line (obtained from Xenogen, Alameda, CA) was used for this study. This cell line has been transfected with luciferase gene and expresses high level of luciferase. Cells were routinely maintained as monolayers in DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 μg/ml) (Invitrogen), and kept at 37°C in humidified atmosphere containing 5% CO_2 in air.

Hyperthermia treatment

For 96-well plate: Cells were seeded in sterile 96-well plates at a density of 1×10^4 cells/well and incubated overnight. For 24-well plate: cells were seeded at densities of 1×10^3, 1×10^4, 1×10^5 and 5×10^5 /well. Hyperthermia was applied at 43°C by sealing the plates with parafilm and enclosing in a Ziploc bag and then immersing the bag into a temperature controlled water bath maintained at 43^o(± 0.1°C). The continuous heating period ranged from 10 to 120 minutes. Controls were sealed in ziplock bags and immersed in a 37°C water bath. After heating, plates were ready for optical imaging, and for MTT and clonogenic assays.

MTT Assay

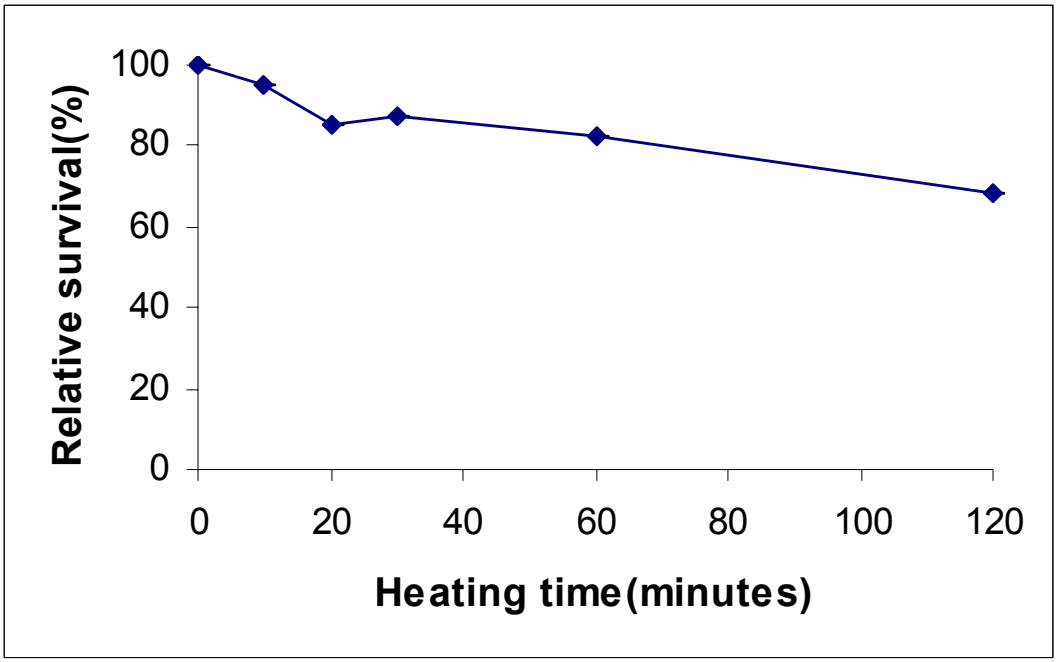
The MTT colorimetric assay was performed to detect tumor cell viability based upon the reduction of the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Chemical Co., St. Louis, MO) by viable cells. This assay detects any reduction of metabolic viability, with or without relationship to apoptosis or necrosis. Immediately after exposure of the cells to 43°C for different times from 10 minutes up to two hours, the old medium was removed, MTT solution (100μl; 0.5mg/ml) in RPMI medium (phenol red free) was added. After incubation for 3 hours at 37°C, cellular mitochondrial dehydrogenase activity reduced the yellow MTT dye to a purple formazan, which was then solubilized with DMSO, the absorbance was determined at a wavelength of 560nm using multiwell scanning spectrophotometer.

For delayed MTT assay, the Cells were incubated at 37^oC for three to five days after the hyperthermia treatment, the cells were returned to 37^oC incubator for three to five days followed by addition of MTT solution (100 μl; 0.5mg/ml) in RPMI medium (phenol red free). The assay was then completed as described above.

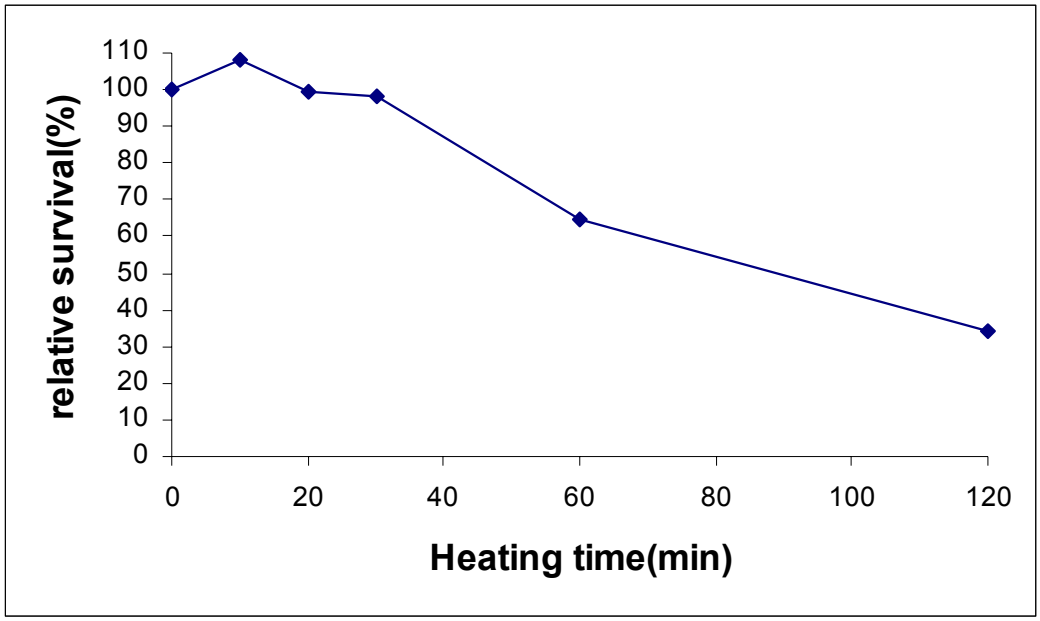
Clonogenic assay

Following hyperthermia treatment, cells were trypsinized at 37°C for 5 minutes, and pipetted up and down 5 times to break up cell clumps and obtain a single cell suspension. The same volume of single cell suspension was plated on 100-mm tissue culture dishes with fresh medium and kept at 37°C, 5% CO_2 incubator for 10~14 days. Colonies consisting of more than 50 cells were counted. Survival curves were generated by plotting percentage rate of the number of colonies formed at a given heating condition to the number of colonies produced by related unheated control cells versus the heating time at the given temperature.

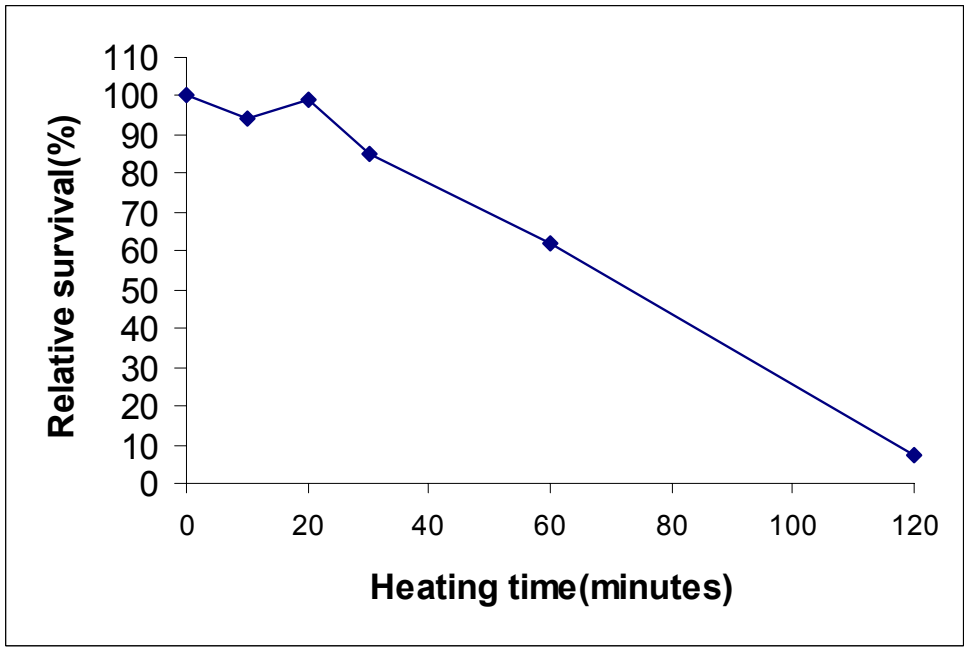
We are trying to adjust the MTT assay protocol with a view to obtaining results that reflect the clonogenic potential of cells.



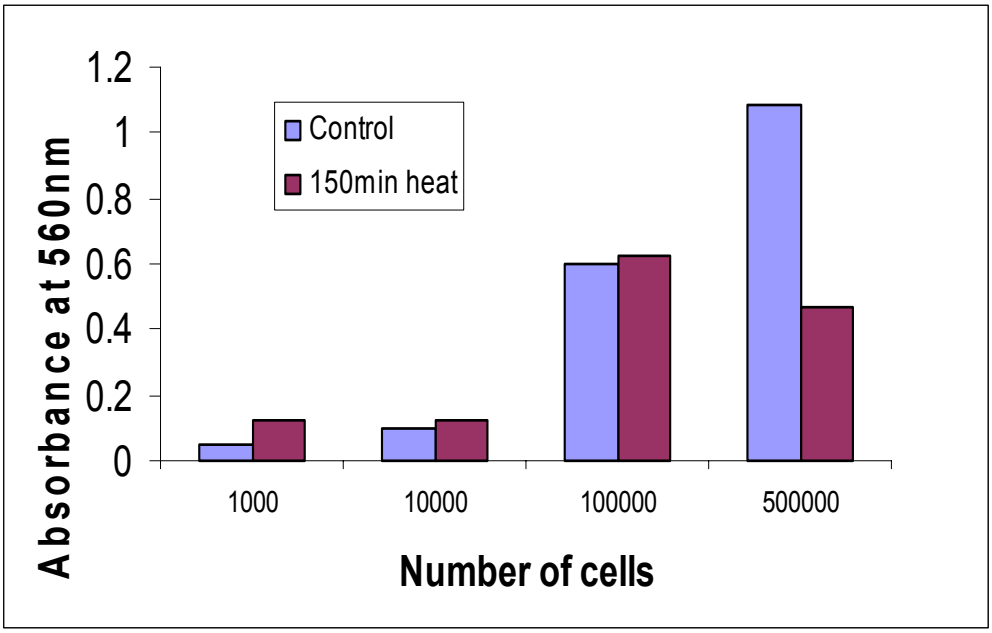
Results of MTT assay performed immediately after heat treatment at 43^0C.



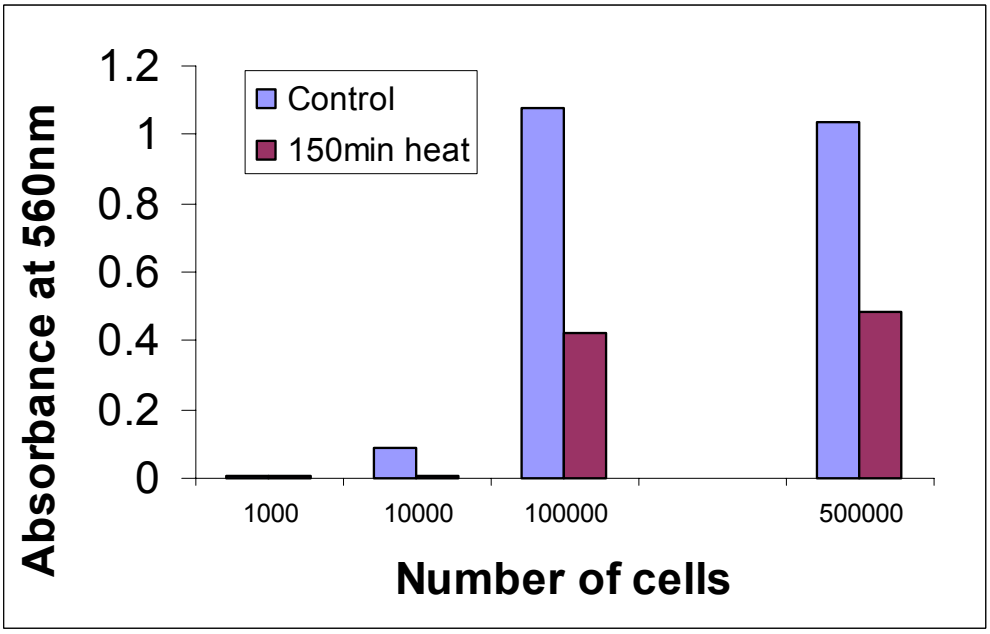
Results of MTT assay performed five days after heat treatment



Cells were subjected to 43^{o} C hyperthermia for different durations and cell survival was determined using clonogenicity assay.



The effect of initial cell numbers on MTT assay carried out immediately after hyperthermis treatment.



The effect of initial cell numbers on the results of MTT assay carried out 5 days after 2 hours of hyperthermis treatment at 43^{o} C.

P43-17: MODULATION OF BISPHOSPHONATE TOXICITY TOWARDS HUMAN BREAST CANCER CELLS IN CULTURE

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Breast cancer has a strong tendency to spread to the bone and 70% of women with metastatic disease have bone involvement. Breast cancer cells produce cytokines, which support the survival of osteoclasts involved in bone erosion. Bisphosphonates are metabolically and chemically more stable analogs of pyrophosphates. Bisphosphonates have an affinity for bone and inhibit osteoclast mediated bone resorption. Therefore, bisphosphonates such as etidronic acid (1-hydroxyethane-1,1,-diphosphonic acid), pamidronic acid (3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid, and zoledronic acid (1-hydroxy-2-imidazole-1-yl-phosphonoethyl) bisphosphonic acid monohydrate) can be used for treating bone metastases and osteoporosis. Clinical trials indicate that bisphosphonates may delay the occurrence of bone metastases and may even have a direct antitumor effect. Bisphosphonates have also shown promise as anti-angiogenic agents. The in vitro cytoxic properties of etidronic acid, pamidronic acid and zoledronic acid towards MCF-7 human breast cancer cells were evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay for cell viability. The 50% inhibitory concentrations were 1230, 87 and 40 micromolar after 72 hours incubation of attached MCF-7 cells with etidronic acid, pamidronic acid and zoledronic acid respectively. The 50% inhibitory concentrations varied somewhat from experiment to experiment, but zoledronic acid was the most potent and etidronic acid was the least cytotoxic. A possible explanation for this variability may lie in the erratic transport of the bisphosphonates into cells in the presence of different positively charged counter ions. When etidronate was combined with putrescine dihydrochloride (1,4-diaminobutane dihydrochloride) or cadaverine dihydrochloride (1,5-diaminopentane dihydrochloride) in the millimolar range, the cytotoxicity towards MCF-7 cells increased slightly. Experiments are in progress to determine if the diamine derivatives putrescine and cadaverine facilitate the transport of etidronate into cells.

The U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0759 supported this work and supported by RCMI grant 2 G12 RR003048, Div. of Infrastructure, NCRR, NIH.

Sridhar R, Kassa A and Ashayeri E. Modulation of bisphosphonate toxicity towards human breast cancer cells in culture. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Abstract P43-17, Philadelphia, PA, 2005. (Abstract)

538 Loperamide sensitizes multidrug resistant MCF-7 clone 10.3 human breast cancer cells to doxorubicin

Yanfei Zhou, Xiaowu Pang, Renshu Zhang, Xinbin Gu, Prabha Kc, Rajagopalan Sridhar.
Howard University, Washington, DC.

Multidrug resistance of tumor cells can limit the efficacy of several anticancer drugs including doxorubicin. There is a need for safe non-toxic agents that reverse multidrug resistance of tumor cells. The anti-diarrhea agent loperamide (4-[p-chlorophenyl]-4-hydroxy-N,N-dimethyl-α,α-diphenyl-1-piperidinebutyramide) was tested as a chemosensitizer of multidrug resistant MCF-7 clone 10.3 human breast cancer cells towards doxorubicin, using the MTT assay for cell viability. The MCF-7 clone 10.3 cells were nearly 200 fold more resistant to doxorubicin than parental wild type MCF-7 cells on the basis of MTT assay. The IC_{50} of doxorubicin decreased from 50 μM to 3 μM and 1 μM respectively, in the presence of 10 μM and 20 μM loperamide, after 72 hours of continuous exposure of MCF-7 clone 10.3 cells to drug(s). Loperamide was slightly toxic to these cells even in the absence of doxorubicin. Treatment of MCF-7 clone 10.3 cells with 10 μM and 20 μM loperamide for 72 hours decreased viability to 80% and 60% relative to untreated control. Flow cytometric analysis of cells treated for 3 hours with doxorubicin (10 μM and 20 μM) with and without loperamide (10 μM and 20 μM), demonstrated enhanced accumulation of doxorubicin by MCF-7 clone 10.3 cells in the presence of loperamide. Cells were treated with loperamide for 3, 24 and 96 hours and analyzed for expression of mRNA of proteins related to drug resistance. The effect of loperamide (10 μM and 20 μM) on the expression of mRNA for the drug resistance related protein MRP in MCF-7 clone 10.3 cells was studied using RT-PCR. The mRNA for MRP decreased even after a 3 hour treatment with loperamide. This decrease persisted over the next four days. Loperamide, which is an over the counter medication for controlling diarrhea, sensitizes MCF-7 clone 10.3 cells to doxorubicin by inhibiting MRP production and increasing doxorubicin retention by cells. This work was supported in part by grant number DAMD17-98-1-8109 from USMRMC and grant 2G12 RR003048 from the RCMI program,NCRR/NIH.

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5863 Cytotoxicity of lipoxygenase inhibitors towards prostate cancer cells in culture

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Free radicals may play a prominent role in the development and progression of prostate cancer. The lipid oxidation product 12-(S)-hydroxy-(5Z,8Z,10E,14Z)-eicosatetraenoic acid (12-S-HETE), which disrupts normal signal transduction in cells, has been associated with hormone refractory prostate cancer. Prostate cancer cells that overproduce 12-S-HETE are relatively resistant to apoptosis. Therefore inhibitors of 12-lipoxygenase may be useful for eradicating hormone refractory cells. The cytotoxicities of non specific lipoxygenase inhibitors such as nordihydroguaiaretic acid (NDGA), coumarin and 4-hydroxycoumarin towards androgen independent human prostate cancer cell lines PC-3 and DU-145 were evaluated using the MTT assay for cell viability. The anthracycline mitoxantrone is used for treating advanced prostate cancer. Therefore the cytotoxicities of the lipoxygenase inhibitors and mitoxantrone were compared. For these studies, attached monolayers of cells were exposed to graded concentrations of NDGA (1 to 20 mM) for either 6 hours or 72 hours, and cell viability was assayed at 72 hours. Cell viability was similar for both 6 and 72 hour treatments with NDGA, suggesting that NDGA was inactivated by metabolism during prolonged exposure to cells. Mitoxantrone toxicity was studied over the micromolar range, whereas the coumarins were tested in the millimolar range. Cell viability decreased to 50, 70, 72 and 64% (relative to control) as a result of 6 hour exposure of PC-3 cells respectively to mitoxantrone (0.1μM), nordihydroguaiaretic acid (20mM), coumarin (1mM) and 4-hydroxycoumarin (1mM). All three lipoxygenase inhibitors were cytotoxic to PC-3 and DU-145 cells, albeit less so than mitoxantrone, which carries some clinical risk of cardiotoxicity. When NDGA (5 mM) was combined with mitoxantrone, it decreased the cytotoxicity of mitoxantrone. Coumarin in the 1 to 5 mM range, was less cytotoxic than 4-hydroxycoumarin towards PC-3 and DU-145 cells after a 6 or 72 hour treatment. The cytotoxicity of coumarin over the 1 to 5 mM range was enhanced when combined with 4-hydroxycoumarin (1 or 5 mM). These results suggest that lipoxygenase inhibitors may have some therapeutic potential without risk of cardiotoxicity. This work was supported in part by grant # DAMD17-03-1-0123 from USMRMC, and grant 2G12 RR003048 from the RCMI program, NCRR/NIH.

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2139 Evaluation of a series of 1,1-dichloro-2,3-di-and tri-arylcyclopropanes as MDR reversing agents.

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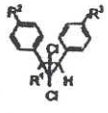
Multidrug resistance of tumor cells can be a problem in cancer therapy with drugs such as doxorubicin (Dox). It is known that the antiestrogen, tamoxifen sensitizes multidrug resistant human breast cancer cells to doxorubicin cytotoxicity. Tamoxifen is not a pure antiestrogen, since it can exhibit some estrogenic activity and increase the risk of endometrial tumors. The pure antiestrogen, Analog II (Z-1,1-dichloro-2,3-diphenylcyclopropane), which was found to be effective as a suppressor of DMBA-induced mammary tumors, was not effective in sensitizing doxorubicin resistant MCF-7/MDR clone 10.3 human breast cancer cells (obtained from M.M Gottesman, NCI). A series of 1,1-dichloro-2,3-di- and 2,2,3-tri-arylcyclopropanes, were evaluated against MCF-7/MDR clone 10.3 cells with respect to their potential for overcoming cellular resistance to doxorubicin. Single cell suspensions of MCF-7/MDR clone 10.3 cells were exposed to graded concentrations of doxorubicin alone and in combination with fourteen antiestrogens at 10 micro molar concentration for three days. Cell viability was assayed in triplicate samples, using the MTT assay and the IC_{50} values for doxorubicin alone and IC_{50} values for doxorubicin in the presence of the cyclopropanes were determined. Comparison of the IC_{50} values gave the extent of reversal of doxorubicin resistance at the 50% survival level. Data for eleven effective compounds are tabulated below (Table). Seven out of the eleven compounds were comparable to tamoxifen in terms of their ability to overcome multidrug resistance. Not listed in the table are Analog II and diethylstilbestrol (DES), which had no effect, as well as dienestrol, which caused 1.6 fold reversal of MDR. These cyclopropyl derivatives have the additional advantage of being pure antiestrogens that may not be associated with the risk of endometrial cancer.

Table: Tamoxifen and 1,1-dichloro-2,3-di-and tri- arylcyclopropanes as MDR reversing agents



Test compound	R^1	R^2	R^3	IC_{50} (μM) for Dox alone	IC_{50} (μM) for Dox in presence of test compound	Fold reversal of MDR
BDRM26	-Ph	$-OCH_2CH_2Br$	-H	53.0	10.0	5.3
BDRM36	$-C_6H_4$-4-OCH_3	$-OCH_2Ph$	-H	53.0	17.5	3.0
BDRM61	$-C_6H_4$-4-OCH_3	-H	-H	53.0	14.0	3.8
BDRM64	$-C_6H_4$-4-OCH_3	$-OCH_2Ph$	$-OCH_3$	53.0	8.0	6.6
BDRM72	-Ph	$-OCH_3$	-H	53.0	9.05	5.9
BDRM81	-Ph	-H	-H	53.0	14.0	3.8
BDRM83	-Ph	$-OCH_2CH_2Br$	$-OCH_3$	53.0	10.05	5.3
BDRM85	-H	$-OCH_2CH_2N(CH_3)_2$	-H	53.0	9.05	5.9
BDRM86	-H	$-O(CH_2)_2N-(CH_2)_3-NH$	-H	53.0	10.05	5.3
BDRM87	-H	$-O(CH_2)_2N-(CH_2)_3$	-H	53.0	9.0	5.9
TAMOXIFEN				53.0	7.01	7.1

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In Vitro Cytotoxicity of Lipoxygenase Inhibitors Towards PC-3 and DU-145 Human Prostate Cancer Cells

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Free radicals may play a prominent role in the development and progression of prostate cancer. The lipid oxidation product 12-S-HETE, which disrupts normal signal transduction in cells, has been associated with hormone refractory prostate cancer. Prostate cancer cells that overproduce 12-S-HETE are relatively resistant to apoptosis Therefore inhibitors of 12-lipoxygenase may be useful for eradicating hormone refractory cells. The cytotoxicity of non specific lipoxygenase inhibitors, nordihydroguaiaretic acid (NDGA), coumarin and 4-hydroxycoumarin towards androgen independent human prostate cancer cell lines PC-3 and DU-145 was studied using the MTT assay for cell viability. The anthracycline mitoxantrone is used for treating advanced prostate cancer. Therefore the cytotoxicities of the lipoxygenase inhibitors and mitoxantrone were compared. For these studies, attached monolayers of cells were exposed to graded concentrations NDGA (upto 20 μM) for either 6 hours or 72 hours. Cell viability was assayed at 72 hours. Cell viability was similar for both 6 and 72 hour treatments with NDGA, suggesting that NDGA inactivated by metabolism during prolonged exposure to cells. Similarly, mitoxantrone toxicity was studied over the micromolar range, whereas the coumarins were tested in the millimolar range. All lipoxygenase inhibitors were cytotoxic to PC-3 and DU-145 cells, albeit less so than mitoxantrone, which carries some clinical risk of cardiotoxicity. When NDGA was combined with mitoxantrone, it decreased the cytotoxicity of mitoxantrone. Coumarin in the 1 to 5 mM range, was less cytotoxic than 4-hydroxycoumarin towards PC-3 and DU-145 cells after a 6 or 72 hour treatment. The cytotoxicity of coumarin over the 1 to 5 mM range was enhanced when combined with 4-hydroxycoumarin (1 or 5 mM). The results suggest that lipoxygenase inhibitors may have some therapeutic potential with perhaps less risk of cardiotoxicity. (This work was supported in part by grant # DAMD17-03-1-0123 from USMRMC, and RCMI grant # 2G1RR003048-16A1 from NIH/NCRR).

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RNA Processing in Health and Disease: A Brief Review

Rajagopalan Sridhar, PhD

Transcription of genes produces mRNA that is subjected to extensive quality control steps involving methylation, polyadenylation, and editing prior to export to the cytoplasm. Misfolded, oxidized, and spontaneously deaminated mRNAs are degraded prior to exiting the nucleus. Alternative splicing of premRNA and extensive editing account for complex and diverse gene expression. Many diseases are associated with aberrant RNA splicing events and under-editing or over-editing of spontaneously deaminated sites in mRNA. This review collates information on a variety of diseases associated with RNA processing defects. An understanding of the mechanisms involved in the pathologic consequences of aberrant RNA processing will be useful in the development of diagnostic tools and treatment strategies.

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Introduction

The central dogma of molecular biology and genetics is that information flows from DNA to RNA, which subsequently dictates protein synthesis. If one considers retroviruses, the dogma also includes the flow of information from RNA to DNA. Four steps represent this important concept. (1) Replication involves semiconservative duplication of double-stranded DNA, with one strand of the double helix serving as template. (2) Transcription is the process by which the information contained in DNA is decoded to produce messenger RNA (mRNA). (3) In eukaryotic cells, the mRNA is processed (mainly by capping, polyadenylation, and splicing) before export from the nucleus to the cytoplasm. (4) Messenger RNA containing the coded instructions is transported to the ribosomes, which decipher the message and synthesize proteins with the correct amino acid sequence and composition. This step is known as translation (*1,2*).

The composition of RNA is similar to that of DNA except that uracil occurs in RNA, whereas its counterpart in DNA is thymine. Moreover, RNA occurs for the most part as single strands, whereas DNA exists predominantly as double strands. The process of translation leading to the formation of proteins using the information contained in mRNA, a series of three nucleotides (codons) code for a particular amino acid or for initiating or stopping protein synthesis. The different amino acids are mobilized to the site of protein synthesis by a series of transfer RNA (tRNA) to form polypeptides. Assuming Beadle's and Tatum's hypothesis of one gene one enzyme, or one gene one polypeptide relationship in the context of the complexity and diversity of human beings compared with less complex and developed organisms, it would be reasonable to expect a proportionately higher number of genes in the human genome. Scientists speculated that the human genome would contain more than 100,000 genes on the basis of expressed sequence tags (ESTs). Completion of the human genome project has shown that there are between 20,000 and 25,000 genes in the human body and that previous estimates based on analysis of ESTs were rather high (*3–9*). It has been pointed out that this is a relatively small number of genes for the complex human being compared with more than 40,000 genes contained in the rice genome. The high complexity and high diversity of proteins in the human being is achieved because of the multifaceted nature of RNA processing, which gives a high degree of flexibility (plasticity) and control.

The ability of a relatively small number of genes in the human to support the varied and complex functions can be explained to a certain extent on the basis of alternative splicing of mRNA (*9*). Considerable chunks of the DNA sequence in the genes of humans and other complex organisms are discarded during the translation of the genetic code and message into functional proteins. The majority of the genes that code for proteins do not exist as contiguous coding sequences (exons) but occur in a fragmented discontiguous fashion, with intervening noncoding sequences (introns). The gene is initially translated into a pre–messenger RNA (premRNA), which is a complementary copy of the exonic and intronic regions. This is then followed by a precisely orchestrated posttranscriptional process of RNA splicing that removes intronic sequences in the premRNA while retaining sequences corresponding to exons in the mature mRNA. As part of the RNA splicing,

exons can be either preserved in the mature message or slated for removal in different permutations and combinations to produce a diverse family of mRNAs from a single premRNA sequence. This process of alternative RNA splicing produces a diverse array of mature mRNAs that can produce different gene products from a single gene encoded in the DNA sequence of the genome. This leads to the production of different isoforms of a protein to be produced, depending on the levels of growth factors, hormone or tissue type (*10–12*). An amazing example of this diversity is a *Drosophila* gene that acts as a precursor for 38,000 different mRNAs. This rather unusual example illustrates the potential of alternative splicing as a mechanism for increasing the versatility of a single gene at the DNA level. Five different types of the structural protein tropomyosin are produced in humans by alternative splicing of the 11 exons of tropomyosin to produce a different tropomyosin in the brain, fibroblast, liver, skeletal muscle, and smooth muscle of humans. This is a far cry from the initial hypothesis by Tatum and Beadle hypothesis of one gene one enzyme, or one gene one polypeptide. Interestingly, the term "RNA world" was coined by Walter Gilbert, who suggested that all life forms originate from RNA in the early stage of evolution. His reasoning was based on the notion that RNA can replicate and act as a catalyst or enzyme and also serve as an information storage molecule (*10–16*).

Alternative splicing accounts for diversity of proteins encoded by the same gene

Alternative splicing in the exons (protein coding region) will produce proteins with different amino acid sequences and functions. If alternative splicing occurs in the noncoding regions of mRNA, then dramatic changes in the expression of proteins may ensue as a result of disruption of the regulatory elements associated with RNA stability domains and translation promoters. RNA splicing is a precisely regulated process that occurs in multicomponent molecular assemblies called spliceosomes, which are sensitive to control by intracellular as well as extracellular signaling pathways. Spliceosomes, which are located in the nucleus, splice out intronic regions from premRNA to produce mRNA, which may subsequently be translated into protein in ribosomes. Spliceosomes recognize intron–exon junctions through splice sequences and several other weak signals for catalytic removal of introns to yield mRNA. Spliceosomes consist of five small nuclear RNAs (snRNAs or U (uridine-rich) RNAs). snRNAs differ from mRNAs in their cap structure involving a special pattern of guanine methylation. Each snRNA is associated with specific factors in a complex assembly referred to as small nuclear ribonucleoprotein particle (snRNP), which contains many proteins. U1 snRNP, U2 snRNP, U4 snRNP, U5 snRNP, and U6 snRNP contain U1 snRNA, U2 snRNA, U4 snRNA, U5 snRNA, and U6 snRNA, respectively. All snRNPs contain proteins B and B′, D1, D2, D3, E, F, and G, which are called Smith (SM) core proteins.

The different snRNPs have specific functions in premRNA processing, which results in the removal of intronic regions prior to the formation of mRNA.

In spite of precise control and regulation of the RNA splicing process, errors are likely, and mRNA surveillance plays an important role in the normal functioning of cells. Proof reading mechanisms ensure the accuracy of DNA replication and RNA transcription. Similarly, proofreading occurs after RNA splicing to monitor the position of the protein stop codon within the mature mRNA and the accuracy of RNA splicing. The protein stop codon is usually located in the last exon of the mRNA. When a stop codon is located upstream of the last exon, the surveillance machinery signals either the degradation of the transcript or the deletion of the premature stop codon to allow the expression of the full-fledged protein. The surveillance machinery causes the destruction of mRNA lacking the stop codon (*17–21*). In the surveillance mechanism termed nonstop decay, the absence of an in-frame stop codon in the mature mRNA leads to transcript degradation (*17*). Nonsense-mediated decay (NMD) detects the premature stop codon in the mRNA and initiates degradation of the mRNA, whereas nonsense-associated altered splicing (NAS) removes the premature stop codon by inducing alternative splicing and allows the production of a nearly complete transcript (*18*).

The insertion of premature stop codons is associated with more than 30% of inherited diseases (*19*). Moreover, many tumors exhibit genomic instability that results in the insertion of premature stop codons. The presence of these premature codons would lead to alternative splicing and consequently different proteins. Such proteins may be valuable as diagnostic markers because of their tumor and disease specificity. Many diseases have been traced to aberrant splicing of mRNA (*22*).

Undoubtedly, alternative splicing of mRNA accounts for the diverse proteins produced from a single gene. In addition to alternative splicing of RNA, there is considerable editing of the RNA by deamination of cytidine to uridine and adenine to inosine at particular sites within mRNA. The enzymes involved in such deamination reactions indicate the potential of mRNA editing to provide further diversity in the expression of a gene. A family of enzymes named cytidine deaminase active on RNA (CDARs) is involved in cytidine-to-uridine conversion at select sites in mRNA. Another

family of enzymes known as adenosine deaminases acting on RNA (ADARs) is involved in RNA editing, which transforms adenosine residues into inosine moieties at select sites within the mRNA. RNA editing is an important component of gene expression (*23–29*).

There are other causes of diversity in the expression of genes. Methylation of DNA and RNA and polyadenylation of RNA can affect gene expression (*30–36*). Perturbation in the methylation of RNA and polyadenylation of RNA can alter the stability of mRNA and contribute to changes in gene expression. One-carbon biochemical pools used in methylation reactions are derived from vitamin B_{12}, folic acid, and S-adenosylmethionine, whereas polyadenylation uses NAD as a building block. Nutritional imbalances with respect to these critical biochemical synthons could affect RNA processing and contribute to health problems. The association of birth defects with folic acid deficiency is traceable to defective RNA processing. The importance of proper RNA processing for normal health and the relationship of aberrant or faulty RNA processing to disease are well recognized (*22,36–41*). Such knowledge may be useful for diagnostic purposes (*39*). Many instances of individual differences in the metabolism and sensitivity to pharmaceuticals can be attributed to the influence of RNA processing on pharmacogenomics (*42–49*). Many clinically used drugs are oxidatively metabolized by CYP2D6, which belongs to the cytochrome P450 (CYP) family of enzymes. Polymorphisms within the CYP2D6 gene accounts for aberrant slicing that results in a truncated inactive enzyme. Many other splicing defects have been reported because of polymorphism within this gene. Between 5% and 10% of Caucasians are unable to metabolize certain pharmaceuticals because they lack adequate levels of functional CYP2D6. Alternative splicing can alter the structure of certain enzymes and receptor molecules. These alterations may influence the response to chemotherapy. Inherited differences in the levels of drug-metabolizing enzymes and receptor targets will also lead to individual variations in patient response to therapy and the occurrence of adverse reactions. Pharmacogenomic influence can lead to alternative spice variants of both proapoptotic (bcl2, bcl-x short, bax, and p53) and antiapoptotic (bad, bcl-x long, MCL1, and survivin) proteins. This can lead to pharmacogenomic differences in response to chemotherapeutic agents that are aimed at the apoptotic machinery. A knowledge of pharmacogenomics can be useful in proper patient selection for clinical trials (*43*).

Examples of diseases associated with aberrant RNA processing

The following are some examples of diseases that are caused by defects in RNA processing.

Beta thalassemia is caused by mutations in the beta globin gene. Aberrant mRNA splicing frequently occurs as a result of mutations in introns 1 and 2 of the beta globin gene. These mutations are associated with this disease, and antisense oligonucleotides have been demonstrated to block the aberrant splice sites. This may provide a therapeutic approach (*50–51*).

Myotonic dystrophy type 2 (proximal myotonic myopathy) is caused by a mutation that produces a pathogenic RNA. This mutant RNA interferes with the proper functioning of the myonucleus at the RNA processing level. This has a toxic effect on muscle fibers and cause myotonic dystrophy (*52*).

A frameshift mutation in the gene for dystrophin causes Duchenne muscular dystrophy. The mutation in question inserts a premature stop codon in the gene and produces a truncated inactive form of the protein dystrophin. Antisense nucleotides have been used to induce alternative splicing of the dystrophin gene to remove the exon containing the premature stop codon and to produce a near-normal length transcript. This restores partial activity and attenuates the severity of the dystrophy (*53–54*).

Spinal muscular dystrophy is characterized by progressive degeneration of spinal cord neurons, leading to paralysis. This disorder is caused by the deletion of the important survivor motor neuron gene (SMN1). The absence of this gene prevents the assembly of critical U1 ribonucleoprotein complexes in the cytoplasm. This leads to errors in premRNA splicing that are associated with this dystrophic condition (*53–54*).

Selective loss of upper and lower motor neurons causes amyotrophic lateral sclerosis (ALS) (Lou Gehrig's disease). Approximately two thirds of individuals with sporadic ALS have a 30% to 95% loss of astroglial glutamate transporter EAAT2 (excitatory amino acid transporter 2) protein in the motor cortex and spinal cord. Several defective EAAT2 mRNAs, including exon skipping and intron retention, have been associated with ALS. In general, aberrant mRNA processing plays an important role in neuron degeneration and excitotoxicity. The presence of the aberrant mRNAs and corresponding proteins in cerebrospinal fluid may be useful for diagnosis of the disease (*55–56*).

Isovaleryl-CoA dehydrogenase (IVD: E.C.1.3.99.10) is involved in the catabolism of leucine. A deficiency of this enzyme prevents oxidation of isovaleryl-CoA to 3-methylcrotonyl-CoA. This can result in neonatal ketoacidosis, which can be fatal to 50% of the afflicted neonates, whereas the others experience developmental delay with or without episodes of acidosis (*47*). IVD is a mitochondrial flavoenzyme of the family of acyl-CoA dehydrogenases (ACDs), which differ in their substrate specificity with respect to the nature and length of the acyl chain (short or medium or long or

TABLE 1 Diseases associated with aberrant RNA processing

Disease or disorder	Gene or protein of interest	Comments
Aceruloplasminemia	Ceruloplasmin	Truncated protein
Alzheimer's disease	Tau	Truncated protein
Albinism, Oculocutaneous 1	TYR	Truncated protein
Amyotropic multiple sclerosis (Lou Gehrig's disease)	EAAT2	
Beta thalassemia	Beta globin gene	Aberrant splicing
Breast cancer . . .	*BRCA1*	
Cystic fibrosis	*CFTR*	Truncated protein
Cystinuria	SLC3A1; SLC7A9	
Dementia, frontotemporal (FTDP-17)	Tau	
Duchenne muscular dystrophy	Dystrophin gene	
Fabry's disease	Alpha-galactosidase A	
Fanconi anemia	FANCG	
Glioma	Several genes	Defective RNA editing. Prone to epileptic seizures
Hemophilia A	Factor VIII	
Leigh's encephalomyelopathy	Pyruvate dehydrogenase E1α	
Marfan syndrome	Fibrillin-1	
Neurofibromatosis type 1	*NF1* neurofibromin	Aberration introduced through CA/EU RNA editing
Ovarian Cancer	*BRCA1*	
Prostate Cancer	KLF6	Enhanced alternative splicing
Sandhoff disease	Hexosaminidase	
Severe combined immunodeficiency	Adenosine deaminase	
Spinal muscle dystrophy	SMN1, SMN2	Aberrant RNA splicing
Tay-Sachs disease	HEXA	Truncated protein, alternative splicing
Von Hippel-Lindau disease type 1	*VHL*	Truncated VHL protein

very long or branched acyl chain). Splicing mutations have been associated with the complex phenotype variations in this disease (*57*).

Enhanced alternative splicing of the KLF6 tumor suppressor gene has been associated with an increased risk for prostate cancer (*41*).

Considerable A-to-I and C-to-U type RNA editing occurs after transcription (*21–23*). Inappropriate editing of RNA can lead to a variety of diseases (*58–61*). The tumor suppressor gene, NF1, associated with neurofibromatosis produces a protein called neurofibromin, which suppresses tumor development and progression. A minor variation in the tumor suppressor gene caused by RNA editing has been identified in neurofibroma tumors. The aberration is introduced through CA/EU RNA editing (*59*).

In gliomas there is a lot of unedited mRNA. Glioma patients are prone to epileptic seizures. In mouse models, the editing process is defective, and epileptic seizures and premature death result (*21–23*).

Diagnostic and therapeutic scope of modulating gene function at the RNA level

RNA interference (RNAi) is a posttranscriptional gene silencing (PTGS) mechanism that is mediated by double-stranded RNA (dsRNA). The dsRNA is processed into small stretches of small duplex RNA moieties of approximately 21 to 22 nucleotides (nts) referred to as small interfering RNAs (siRNAs) by an enzyme called Dicer (an RNAse III). A multiprotein complex known as RNA-induced silencing complex (RISC) interacts with siRNAs. This interaction results in a sequence-specific association of activated RISC complex with the recognized RNA transcript and leads to sequence-specific scission of the transcript. RNAi is useful for unraveling the relationship of gene function in cancer and infectious diseases (*62–63*).

Alterations in the ratio of RNA isoforms of fibroblast growth factors (FGFR2) have been correlated with lung, skin, and bone defects (*64–66*) Polypyrimidine tract binding protein (PTB) is involved as an exon silencer in the control of alternative splicing of FGFR2. This is thought to play a role in cancer progression (*65*).

Antisense oligoribonucleotides can be used to correct splicing defects and restore expression of the normal or nearly normal protein. This offers an attractive therapeutic strategy (*66–70*). Similarly, it may be possible to eliminate harmful exons. Exon-specific splicing enhancement by small chimeric effectors (ESSENCE) in connection with the BRCA1 breast cancer gene and the SMN2 gene for spinal muscular atrophy are examples that come to mind. Many human genetic diseases result from point mutations that produce aberrant splicing. These mutations may affect splice site sequences directly, or they may perturb regulatory elements such as exonic splicing enhancers (ESE), which are binding sites for serine-arginine-rich (SR) splicing factor proteins. Fusing minimal RS domains of SR

splicing factor proteins to antisense oligonucleotides produces effector molecules that can be used to target exons that may have been skipped because of mutations. This is a strategy for overcoming the negative effects of a mutation in an exon (*71–73*). It must be mentioned that in the attempt to eliminate an isoform associated with a disease, there could be adverse effects if the particular isoform also has an important normal function unrelated to the disease.

Alternative splicing of therapeutic and diagnostic importance affects apoptosis by producing isoforms of tumor suppressor gene products and by producing antiapoptotic products derived from splice variants. For example, bcl-xl is antiapoptotic, and high levels of this protein in tumors render them refractory to chemotherapy. An antisense oligonucleotide for shifting splice variants from the antiapoptotic bcl-x long to the bcl-x short isoform may increase the sensitivity of tumor cells to chemotherapy. In the attempt to eliminate an isoform associated with a disease, there is the possibility of adverse effects if the eliminated isoform also has an important normal function unrelated to the disease AU4 state.

Significant levels of oxidized mRNA were found in the frontal cortex region of the brains removed from Alzheimer patients at autopsy . The oxidized mRNA may lead to abnormal proteins and cause neuronal death. Oxidative damage to mRNA was not random, because certain mRNAs were more prone to oxidative damage (*75,76*). There are also reports of oxidative damage to ribosomal RNA (*77,78*).

Two human transcription factors that are related to rRNA transferases involved in mitochondria appear to be associated with maternally inherited deafness.

Erroneous coupling of transcription and translation at the inner mitochondrial membrane may hold clues to the cause of a form of Leigh syndrome (*74*). Processing of mitochondrial RNA may be important in maternally inherited diseases. Polyadenylation of mitochondrial RNA can stabilize mitochondrial RNA. Undoubtedly, RNA processing in mitochondria would have important consequences for mitochondrial activities such as cellular energy production and regulation of apoptosis and calcium homeostasis. These aspects are not covered in this review.

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